The Identification of Inhibine

The use of honey as a wound-healing and antiseptic agent has been recorded since ancient times. Stomfay-Stitz (1960) has reviewed this subject in his interesting article, "Honey: An Ancient Yet Modern Medicine." Examples of this use of honey can be found in the modern literature (Bulman, 1955; Seymour and West, 1951).

The presence in honey of an antibacterial activity was first shown by Dold, Du and Dziao (1937) about 25 years ago. Their work was confirmed in several other laboratories (Prica, 1937; Plachy, 1944) and in the intervening time several other papers have appeared on the subject. It has been generally agreed that "inhibine" (the name used by Dold for the antibacterial activity) is sensitive to heat and light, and the effect of heating of honey on its inhibine content has been studied by several investigators, including Dold, et al. (1937), Prica (1937), Schade, Marsh and Eckert (1958), Duisberg and Warnecke (1959), and Stomfay-Stitz and Kominos (1960). It is apparent that heating of honey sufficiently to reduce markedly or destroy its inhibine activity would deny it a market as first-quality honey in several European countries. In fact, Duisberg and Warnecke proposed the use of sucrase and inhibine assays together to determine the heating history of commercial honey. Until the present, no information on the actual nature or constitution of inhibine has been published.

During our work on an enzyme in honey that produces acidity it was shown that the acid formed is gluconic acid and that the enzyme responsible is a glucose oxidase. Details of the identification of this enzyme will appear in a forthcoming publication. Glucose oxidase has been known to occur in molds and some years ago Gauhe (1941) demonstrated that an enzyme oxidizing glucose to gluconic acid and producing hydrogen peroxide occurred in the pharyngeal gland of the honey bee. It has not been reported in honey.

Enzymes of this type produce hydrogen peroxide during their action on glucose according to the equation:

$$\text{glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconolactone} + H_2O_2$$

The gluconolactone can further change to gluconic acid, which can exist in equilibrium with the lactone. The enzymic production and accumulation of hydrogen peroxide in diluted honey accounts for all of the reported properties of inhibine and we conclude that the antibacterial activity of honey as demonstrated in the inhibine assay is actually due to hydrogen peroxide. The work upon which this conclusion is based is summarized below and reported in detail elsewhere (White, Subers, and Schepartz, 1962).

A crude enzyme preparation from honey made by alcohol precipitation and dialysis was found to produce hydrogen peroxide when incubated with glucose, by testing with peroxidase and a dye (o-dianisidine) which becomes colored in the presence of hydrogen peroxide and peroxidase. The depth of color (peroxide produced) was proportional to the concentration of the honey enzyme. Paper chromatography of the enzyme-glucose solution showed that an acid similar to gluconic acid was produced; a comparison of infrared spectra showed it to be gluconic acid.

The assay procedure for inhibine in honey was described by Dold and Witzenhausen (1955) and slightly modified by Schade et al. (1958). As modified it requires the preparation of five nutrient agar petri plates which contain graded amounts of diluted honey, so that the honey concentration in the plate series runs from 20% in the first plate to about 4% in the fifth plate. The surfaces of the nutrient media in the plates are inoculated with a fresh culture of the organism *Staphylococcus aureus*, incubated at 37°C. for 24 hours, and then examined. The inhibine number is taken as the number of the plate with the lowest honey concentration that does not permit any visible growth of the bacteria. For example, a number of 0 means growth on all plates; 3 means no growth on the third (12.5% honey) plate and growth on the fourth (8.5%) and fifth (4.3%) plates. An inhibine number of 5 indicates that none of the plates allowed bacterial growth.

A procedure was devised by which we could measure the amount of hydrogen peroxide produced in these agar plates during the standard inhibine assay. This was done by adding peroxidase and dianisidine to the medium, resulting in a gel that darkened in proportion to the amount of peroxide present.

Eight honey samples were assayed for inhibine by the standard procedure and the hydrogen peroxide production rate was determined in a parallel series of plates. It was found that all plates that did not allow growth of the inoculum in 24 hours showed a peroxide production from the honey present of at least 35 micrograms per plate in two hours. This is shown in Table 1. Plates allowing growth produced 26 or less micrograms in two hours. When small amounts of hydrogen peroxide were added to inhibine assay plates containing inactive boiled honey, no

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TABLE 1
Relation Between Peroxide Production Under Assay Conditions and Inhibine in Honey

Honey	Plate Inhibiting Growth		Plate Allowing Growth	
	No.[1]	Peroxide[2]	No.	Peroxide[2]
Aster	4	117	5	16
N. E. Fall flower	4	125	5	17
Goldenrod-Aster	4	140	5	19
Tupelo	3	84	4	26
Clover	0		1	22
Cotton	5	65	0	
Alfalfa	3	44	4	22
Clover	2	35	3	20

[1]Corresponds to inhibine number of sample.
[2]Micrograms Hydrogen Peroxide produced per plate at 2 hrs.

growth took place in plates that contained about 30-45 micrograms of peroxide after 24 hours incubation; those allowing growth had less than about 15 micrograms of peroxide at that time.

It was then demonstrated that the destruction of hydrogen peroxide as it was formed in the honey assay plates allowed growth on all plates and reduced the apparent inhibine number to zero. This was done by including catalase (an enzyme that destroys hydrogen peroxide) in the plates. By inoculating with *S. aureus* the set of plates in which the peroxide was destroyed by the measuring system of peroxidase and dianisidine, growth was also observed at 24 hours in all plates. The results are shown in Table 2.

When the honey enzyme preparation described above was assayed it gave an inhibine number of zero and showed no peroxide production. When the assay was repeated with the addition of glucose in amounts normally present from honey in the assay, considerable peroxide was produced but growth was present though reduced in all plates, so the inhibine number was still zero. Addition of fructose, which is not acted on by glucose oxidase, or substitution of inactive dried honey for glucose and fructose produced an inhibine number of 3. None of the sugars or dry honey showed any inhibine activity or peroxide production in the absence of the enzyme preparation. These results, together with other observations, show that in the inhibine assay procedure for honey, an osmotic factor is present, which has the effect of reducing the amount of hydrogen peroxide required to prevent growth on the inoculated and incubated medium by an amount related to the honey concentration. Above a certain level of honey concentration all honeys will inhibit growth of the organism due to the osmotic effects of the sugars. This level is of course higher than that of the first assay plate.

During this work it was noted that honey destroys hydrogen peroxide, at a rate slower than that of the measuring system used. The antibacterial effect of honey, as demonstrated by the inhibine assay, will depend not only on the rate of production of peroxide, which is a function of enzyme activity, but in addition will be affected by the rate of destruction of peroxide by honey components, which probably also varies among honeys. The other product of the enzyme reaction, gluconic acid (or more properly, gluconolactone), does accumulate in honey.

Attempts made to detect and measure hydrogen peroxide in undiluted honey failed; less than 0.3 microgram per gram was present.

Previous work on the enzymic production of acid in honey during storage (White, Riethof and Kushnir, 1961) showed a rate equivalent to about 0.002 microgram of hydrogen peroxide per gram of honey per hour. When honey is diluted, however, production rates increase 2500-50,000 times to those found in this work, from 5 to nearly 100 micrograms per gram per hour. This enzyme is being studied further. Full experimental details and results obtained are included in an article which has been submitted to a technical journal.

TABLE 2
Effect of Destruction of Hydrogen Peroxide During Assay on Inhibine Value in Honey

Plate No.	*Growth of S. aureus in Plate Containing*		
	Catalase	Peroxidase-Dianisidine	Neither
1	++	+	−
2	++	++	−
3	++	+++	−
4	+++	+++	−
5	+++	++++	++
Inhibine No.	0	0	4

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